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Mutation of Nucleotide 1,762 in the Core Promoter Region During Hepatitis B e Seroconversion and Its Relation to Liver Damage in Hepatitis B e Antigen Carriers

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In chronic hepatitis B virus (HBV) infection, mutations develop frequently at nucleotides 1,762/1,764 in the X protein open reading frame, where the core promoter is also located. By using a modified allele-specific polymerase chain reaction method, the longitudinal emergence of the A→T mutation at nucleotide 1,762 was studied in relation to precore mutations, genotype, and liver damage.

First, samples from 38 carriers that were drawn before and after hepatitis B e (HBe) seroconversion were tested. T-1,762 mutant strains increased during HBe seroconversion (P = 0.004). In the HBe antigen-negative ($HBeAg^-$) phase, T-1,762 mutants were found in 71% (12 of 17) of patients without compared with 33% (6 of 18) of patients with a concomitant precore mutation that prevents HBeAg synthesis (P = 0.08).

Second, in 55 $HBeAg^+$ patients, the T-1,762 mutant was found to be associated with more liver inflammation (P = 0.04) and fibrosis (P = 0.02), as measured by histology activity index (HAI) scores.

The results show that the nucleotide (nt) 1,762 A→T mutation often develops during HBe seroconversion, particularly in strains without precore mutations that prevent HBeAg production. For unknown reasons, the T-1,762 mutant was rare in genotype B strains. The presence of a T-1,762 mutant in the $HBeAg^+$ phase may be useful for identifying immunoactivation in previously immunotolerant carriers, which could be valuable for selecting patients for interferon therapy. ***J. Med. Virol. 55:185–190, 1998.***

KEY WORDS: hepatitis B e antigen; core promoter; histology activity index; mutation

INTRODUCTION

Hepatitis B e antigen (HBeAg) can be detected in chronic hepatitis B as a marker of productive infection. Loss of HBeAg indicates a reduced viral replication and infectivity as a result of the host's immune response. However, loss of HBeAg is not a true reflection of diminishing viral replication but, in some cases, merely a sign of viral escape by a G→A mutation at position 1,896, which creates a premature TAG stop codon and abolishes the synthesis of HBeAg [Brunetto et al., 1989; Carman et al., 1989]. It has been shown that the mutation at nucleotide (nt) 1,896 emerges frequently during HBe seroconversion in hepatitis B virus (HBV) strains with thymine but emerges rarely in strains with a cytosine at nt 1,858 [Li et al., 1993; Lok et al., 1994; Lindh et al., 1995, 1996].

Recently, mutations in the core promoter were suggested to mediate down-regulation of HBeAg production [Okamoto et al., 1994] and to account for fulminant hepatitis from infection by HBV without the precore TAG mutation [Sato et al., 1995]. The predominant mutation in these and other reports [Nishizono et al., 1995; Takahashi et al., 1995; Kidd-Ljunggren et al., 1997] was a double mutation at positions 1,762–1,764, changing AGG to TGA. This mutation was proposed to act through effects on a putative HBe TATA box [Okamoto et al., 1994], but subsequent studies showed the absence of a TATA box at this location [Chen et al., 1995] and failed to find any impact on transcription [Nishizono et al., 1995]. However, a more recent study showed that the 1,762–1,764 muta-

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tions do reduce the transcription of precore mRNA by interfering with the binding of transcription factors, thus supporting the finding that these mutations down-regulate HBeAg synthesis [Buckwold et al., 1996].

The aim of the study was to analyze the development of the nt 1,762 mutation during HBe seroconversion in carriers with or without emerging precore TAG mutants and to determine how these events are related to HBV genotypes. The nt 1,762 mutation was also studied in relation to liver damage in $HBeAg^+$ patients. For these purposes, a method for point mutation analysis was developed based on coamplification of wild type and mutant DNA by competitive primers and a subsequent separation of the polymerase chain reaction (PCR) products by restriction enzyme action.

MATERIALS AND METHODS

Patients and Histopathology

Two categories of patients were studied. First, serum samples were examined from $HBeAg^+$ and $HBeAg^-$ phase in 38 carriers in whom the emergence of precore mutations had been studied previously [Lindh et al., 1995]. The patients were grouped on the basis of precore variability: group A with C-1,858 strains, group B with T-1,858 strains but without a precore TAG mutation after HBe seroconversion, and group C with T-1,858 strains and TAG mutants emerging during HBe seroconversion.

Second, we analyzed serum samples from 59 $HBeAg^+$ carriers whose liver damage was assessed by histology activity index (HAI) scoring [Knodell et al., 1981] in a blinded fashion. In these cases, nt 1,858 and nt 1,896 had been analyzed in a previous study [Lindh et al., 1996]. Comparing histopathology, we used the fibrosis score (HAI_{fibr}) and the sum of the component scores for piece-meal necrosis, lobular inflammation, and portal inflammation (HAI_{infl}) separately.

Serology

HBeAg and anti-HBe were tested by using Abbott IMX (Abbott Laboratories, Abbott Park, IL).

Genotyping

A genotyping method based on restriction fragment-length polymorphism (RFLP) analysis of an S-gene amplicon [Lindh et al., 1997] was applied on $HBeAg^+$ samples. Briefly, this method amplifies the genomic segment between nt 256 and nt 796 in the S region by using PCR, and, after a subsequent incubation with Hinf1 and Tsp509I, the genotype is obtained from the RFLP patterns on agarose electrophoresis.

Analysis of Nt 1,762

Typically, there is an AGG→TGA double mutation at nt 1,762–1,764 [Okamoto et al., 1994]. To detect this mutation, we developed a modification of allele-specific PCR [Lo et al., 1992], which we termed competitive primer-carried restriction site (CPRS) analysis. The method discriminates between A (wild type) and T (mutant) at nt 1,762. Two antisense primers matching a wild type [P-WT; nt 1,789–1,762; 5′TGCCTACAGCCTCCTA(A/G)TACAAAGGCCT] and a mutant [P-MUT; nt 1,789–1,762; 5′TGCCTACAGCCTCCTAATACAAA(A/G)ATCA] template were used in the same PCR reaction, thus, competing with one another in the annealing step (A/G indicates a mixture of A and G). Because the antisense primer matching the wild type HBV (P-WT) carries an Stu1 site (underlined), a PCR product with an Stu1 site at the downstream end would be expected if the sample contains A-1,762. Conversely, in PCR amplifying a T-1,762 mutant strain, the primer P-MUT has a better 3′ match and, by competition, prevents amplification by P-WT, thus, creating a PCR product without the Stu1 site (Fig. 1). Forty cycles of amplification using an annealing temperature of 55°C and 1 μl of each of primer's P-sense1 (nt 1,596–1,625; 5′TCTGCACGTCGCATGGAGGCCTCCGTGAAC),

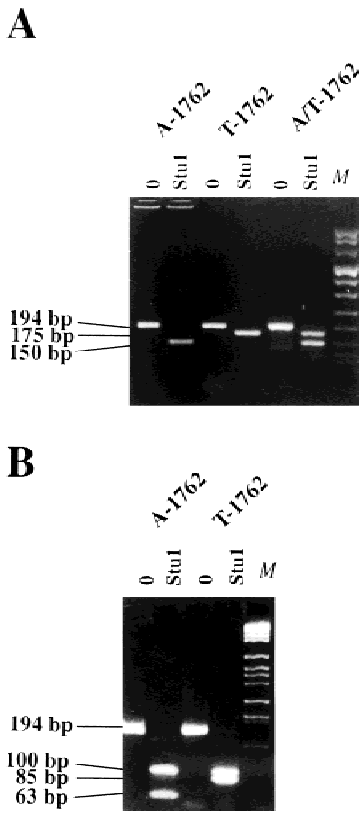

Fig. 1. Agrose gel electrophoresis showing representative patterns from competitive primer-carried restriction site (CPRS) analysis of nucleotide (nt) 1,762 of hepatitis B virus (HBV). Each sample is run in two lanes: On the left, the untreated polymerase chain reaction (PCR) product is shown; on the right, the same product is shown after digestion with Stu1. A-1,762, wild type; T-1,762, mutant; A/T-1,762, mixture of wild type and mutant strains. **A:** Analysis of the PCR fragment nt 1,596–1,762. Stu1 treatment cuts the PCR fragment in both ends, resulting in a band shift from 194 base pairs (bp) to 150-bp size. In T-1,762 strains, the PCR fragment is cut only in the site carried by the sense primer (serving as Stu1 efficiency control), resulting in a band shift to 175-bp size. **B:** Analysis of the PCR fragment nt 1,603–1,789 (genotype A). The pattern is different due to an additional Stu1 site at nt 1,703: After Stu1 incubation, in addition to a 100-bp band (1,603–1,702), A-1,762 strains produce a 63-bp band (1,703–1,765), and T-1,762 strains, instead, produce an 85-bp band (1,703–1,789).

P-WT and P-MUT. After PCR, 10 μl of the amplified product were incubated at 37°C overnight with 1 μl of Stu1, 1.5 μl of buffer, and 2.5 μl of water. The samples were then run on gel electrophoresis in parallel with 10 μl of PCR product mixed with 1.5 μl of buffer and 3.5 μl of water but without enzyme.

Primer P-sense1 carries an internal Stu1 site (underlined). Incubation of the PCR product with Stu1 therefore detaches a 19-base-pair (bp) fragment at the upstream end of all the PCR products and a corresponding shift of the DNA band, serving as a control for the Stu1 action efficiency (Fig. 1). HBV of genotype A was found to have an additional, naturally occurring Stu1 site at nt 1,703, producing a different pattern, which, in mutant strains, produced overlapping 1,615–1,702 and 1,703–1,789 bands, making detection of mixed infection difficult. By using primer P-sense2 (nt 1,603–1,625; 5′GTTGCATGGAGACCACCGTGAAC; not carrying the internal control Stu1 site) as sense primer in the genotype A samples, this overlap was avoided (Fig. 1).

In HBeAg$^-$ samples, a nested procedure was used. First, 30 cycles of PCR were run using P-sense1 and P-antisense-outer (2,154–2,125; 5′ATGGGATCCCTGGATGCTGGGTCTTCCAAA) and an annealing temperature of 55°C. This was followed by 35 cycles of a second PCR, as described above (P-WT and P-MUT together with P-sense1 or P-sense2).

Confirmation of CPRS Accuracy

A segment of HBV DNA from genotype A isolates carrying AGG and TGA, respectively, at nt 1,762–1,764 was inserted at the Hinc2 site of pUC18 after amplification by PCR using the primers 5′GAGGACTCTTGGACTCTCAGCAATGTCAAC (nt positions 1,658–1,687) and 5′CTGAGTTTTAGGCCCATATTAACGTTGACA [nt positions 2,193–2,164; the Hinc2 sites, GT(C/T)(A/G)AC, are underlined]. Cloned pUC18 carrying the HBV DNA insert was purified and quantified spectrophotometrically, and appropriate mixtures of mutant and wild type DNA were subjected to CPRS analysis by using 5′ATGTCGACAACCGACCTTGA (nt positions 1,680–1,699) as sense primer (Fig. 2). To challenge specificity, a high concentration (10^9 copies/ml) of the mixture was used.

Sequencing

Sixteen samples were examined by direct sequencing with chain termination using the Sequenase kit (US Biochemical Co., Cleveland, OH), as described previously [Lindh et al., 1995].

RESULTS

CPRS Technique

The results from CPRS analysis of mixtures with known ratios of mutant/wild type HBV DNA are shown in Figure 2. The accuracy of the method was established further by sequencing HBV DNA from 16 serum samples, with no case showing different results (Table I).

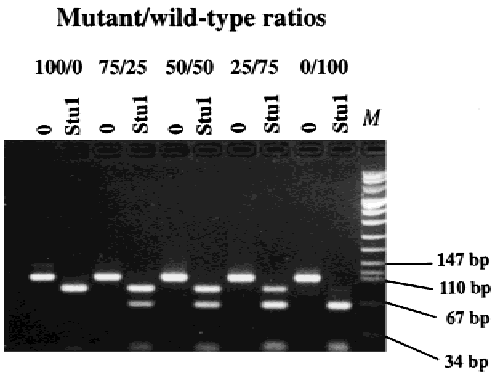

Fig. 2. CPRS detection of known ratios of 1,762–1,764 TAG mutant/AGG wild type mixtures of genotype A HBV DNA cloned into pUC18. Each mixture is represented by two lanes: the first (0) with untreated 110-bp amplicon (nt 1,680–1,789) and the second (Stu1) with the same product after incubation with Stu1. Mutant HBV then produces an 85-bp band, and wild type HBV produces a 63-bp band. A faint mutant band appears in the 0/100 lanes, representing weak, unspecific annealing due to high template concentration.

Seroconverting Patients

In the HBeAg$^+$ phase, an A-1,762 wild type strain was present in 87% (33 of 38) of the samples (8 with A/T-1,762 mixtures) compared with 56% (19 of 34; 3 with A/T mixtures) in the first HBeAg$^-$ sample (Table I; $P = 0.004$; Fisher's exact test).

Eleven of 13 carriers in group A (C-1,858) had an A-1,762 strain in HBeAg$^+$ stage, 4 with a mixture with T-1,762. After HBe seroconversion, 8 of 12 carriers who were CPRS-PCR$^+$ had T-1,762 exclusively; the remaining 4 showed A-1,762 exclusively. Of note, 1 of these 4 carriers had *a strain with* a precore start codon mutation (identified by sequencing), and 1 became HBeAg$^+$ during a reactivation several years later.

In group B (T-1,858 without precore TAG mutation), 6 of 7 patients had an A-1,762 strain in the HBeAg$^+$ sample (2 with A/T mixtures) compared with 3 of 6 in the HBeAg$^-$ phase (2 with A/T mixtures). In group C (T-1,858 with a precore TAG mutant after HBe seroconversion), 16 of 18 HBeAg$^+$ samples had an A-1,762 strain. In this group, 12 of 16 still had an A-1,762 strain after HBe seroconversion, and only 5 had a T-1,762 mutant (one with an A-1,762/T-1,762 mixture).

Thus, of the 17 CPRS-PCR$^+$ patients in group A and B who did not present a precore mutation after HBe seroconversion (i.e., all except patients A7 and A8; see Table I), 12 (71%) had exclusive ($n = 10$) or partial ($n = 2$) T-1,762 mutant strains in sample 2. In comparison, of the 18 carriers that showed a precore mutation in sample 2 (2 in group A and 16 in group C), 6 (33%) had an exclusive ($n = 5$) or partial ($n = 1$) 1,762 mutant strain ($P = 0.08$; Fisher's exact test). Of 18 CPRS-PCR$^+$ follow-up samples, 16 showed unchanged viral populations, and 2 showed an increased mutant fraction compared with the first sample after HBe seroconversion.

Genotype Impact

Most patients with HBV of genotype A (with C-1,858) remained with a precore wild type strain (Table I). In

TABLE I. Demographic and Serologic Data and Results of the Nucleotides 1,858, 1,896, and 1,762 of Hepatitis B Virus DNA*

				HBeAg+			HBeAg−		Follow up[a]	
No.	Age/sex	Origin	Genotype	Nt 1,858	Nt 1,896	Nt 1,762	Nt 1,896	Nt 1,762	Nt 1,896	Nt 1,762
A1	24/M	Sweden	A	C	G	A	G	A	G	A
A2	38/F	Sweden	A	C	G	A	G	T	G	T[b]
A3	24/M	Sweden	A	C	G	A/T	G	T	G	T[b]
A4	37/M	Sweden	A	C	G	A	G	neg	neg	neg
A5	42/M	Sweden	A	C	G	T	G	T[b]	G	T
A6	53/M	Sweden	A	C	G	A	G	A	G[c]	A
A7	29/M	Sweden	A	C	G	A[b]	G	A	G[d]	A
A8	43/M	Sweden	A	C	G	A/T[b]	G/A	T	neg	neg
A9	24/M	Arab Emirate	A	C	G	A/T	G	T	G	T[b]
A10	31/M	Thailand	C	C	G	A	G	T	G	T
A11	12/M	Vietnam	C	C	G	A	G	A	neg	A
A12	12/M	Vietnam	C	C	G	A/T	G	T	G	T
A13	12/F	Vietnam	UT	C	G	T	G	T	na	na
B1	37/F	Sweden	C	T	G	A/T	G	A/T	neg	neg
B2	14/M	Ethiopia	D	T	G	A[b]	G	A/T	neg	neg
B3	13/F	Turkey	D	T	G	A	G	A	neg	neg
B4	29/M	Iran	D	T	G	A	G	neg	neg	neg
B5	4/F	Korea	C	T	G	T	G	T	neg	neg
B6	12/M	Korea	C	T	G	A	G	T	G/A	T
B7	34/F	Korea	C	T	G	A/T	G	T[b]	na	na
C1	40/M	Sweden	D	T	G/A	T	G/A	T	G/A	T
C2	46/M	Sweden	D	T	G	A	G/A	A[b]	G	A
C3	31/M	Yugoslavia	D	T	G/A	A/T	G/A	T	G/A	T
C4	43/M	Yugoslavia	D	T	G	A	A	T	na	na
C5	32/M	Togo	E	T	G	A	A	neg	na	na
C6	17/M	Gambia	E	T	G	A	G/A	A	G/A	neg
C7	25/F	Gambia	E	T	G	A[b]	G/A	A	G/A	A/T
C8	11/F	Iran	D	T	G	A	A	A	na	na
C9	25/M	Turkey	D	T	G	A	G/A	A	G/A	A[b]
C10	26/M	Turkey	D	T	G/A	A/T[b]	G/A	A/T	A	T[b]
C11	18/M	Syria	D	T	G	A	G/A	A	A	neg
C12	14/M	Lebanon	D	T	G	A	G/A	A	na	na
C13	17/M	Taiwan	C	T	G	T	G/A	T	neg	neg
C14	11/F	Vietnam	B	T	G	A	A	neg	A	neg
C15	26/F	Vietnam	B	T	G	A[b]	G/A	A	A	A
C16	34/F	Vietnam	B	T	G	A	A	A	na	na
C17	30/F	Vietnam	UT	T	G	A	G/A	A	na	na
C18	29/M	Vietnam	UT	T	G	A	G/A	A	G/A	neg

*Samples from HBeAg+ and HBeAg− phase were analyzed. The wild type sequence at 1,762–1,764 is AGG, whereas essentially all T-1,762 mutants have TGA. Age: years of age at the time of sample 2; Nt: nucleotide (position from the unique EcoRI site); HBeAg: hepatitis B e antigen; antiHBe: antibody to hepatitis B e antigen; M: male; F: female; G: guanine; C: cytosine; A: adenine; T: thymine; A/T: mixtures with an estimated fraction of >25% of the minor strain (i.e., the T-1762 strain in most cases); UT: untypable; neg: negative by this PCR; na: no sample available.
[a]Mean 3.5 years, range 0.9–7.2 years.
[b]Confirmed by sequencing.
[c]Reappearance of HBeAg.
[d]Sequencing showed precore start codon mutation.

addition, three carriers had genotype C strains with C-1,858 and did not develop a TAG mutation. The number of patients was too small to calculate the correlation between genotype and emergence of promoter mutations in the seroconverting patients. However, out of the 9 East Asian seroconverting carriers with established genotype, 0 of 2 with genotype B compared, with 6 of 7 with genotype, C presented a T-1,762 mutation. Inclusion of East Asian carriers in the biopsy group showed that 12 of 18 with genotype C (four with C-1,858), compared with 0 of 7 with genotype B, showed a T-1,762 strain ($P = 0.01$; Fisher's exact test).

Nt 1,762 and Liver Damage

Fifty-five of the 59 HBeAg+ patients were positive by CPRS-PCR. Thirty-seven patients had an A-1,762 wild type strain, and 18 had a partial (n = 10) or an exclusive (n = 8) T-1,762 mutant. Carriers with mutant strains showed more liver damage than those with wild type strains ($P = 0.02$ for HAI_{fibr}; $P = 0.04$ for HAI_{infl}; Mann-Whitney rank-sum test; Fig. 3).

DISCUSSION

In this study, we found that an A→T mutation at nt 1,762 often emerges before or during HBe seroconversion and that the presence of this mutation in HBeAg+ carriers indicates immune activation and liver damage.

In the immunoactive phase of chronic HBV infection, mutations that alter the presentation of target antigens may be advantageous for the virus and, thus, may contribute to viral persistence by immunological escape. Recent studies have demonstrated that a muta-

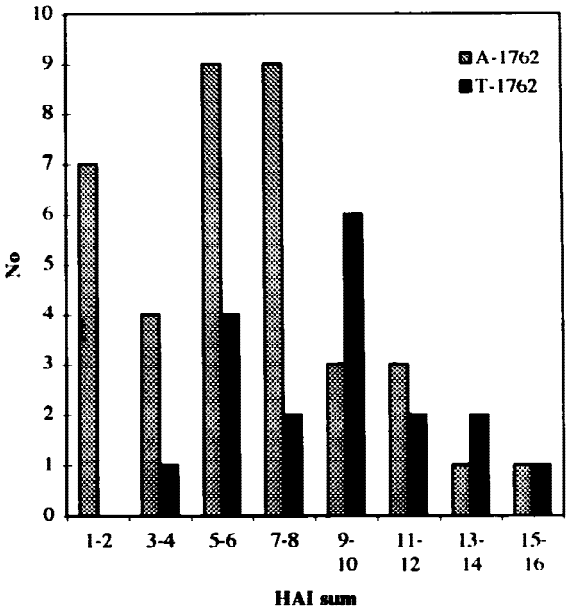

Fig. 3. Histology activity index (HAI) scores (HAI_{sum}; the sum of the inflammation and fibrosis components of HAI) in 55 hepatitis B e antigen-positive ($HBeAg^+$) carriers infected with A-1,762 or T-1,762 strains of HBV. The T-1,762 bars include carriers infected with T-1,762 strains exclusively (n = 8) or with mixtures of A-1,862 and T-1,762 (n = 10).

tion at position 1,762 is present in a large proportion of $HBeAg^-$ carriers. This mutation resides in a region that harbors both the core/precore promoter (the basal core promoter; BCP) [Yuh et al., 1992] and codes for the amino acids of the X protein.

We found that a wild type A-1,762 strain was present in 89% of the samples in the $HBeAg^+$ phase compared with 57% after HBe seroconversion ($P = 0.004$), indicating that essentially all chronic HBV infections start with a promoter wild type strain and that mutants emerge as a result of immune activation during HBe seroconversion. The T-1,762 mutation was found in 71% of carriers in whom a precore TAG mutation did not emerge compared with 33% of the carriers with a TAG mutation in $HBeAg^-$ stage ($P = 0.08$). This indicates that this mutation may develop more frequently in strains without precore mutations, thus supporting the concept that immune escape by a reduced HBeAg production may be achieved not only through precore mutation (acting at the translational level), but also by promoter mutations that reduce transcription of precore mRNA. The original hypothesis that this was mediated by effects on a putative TATA box [Okamoto et al., 1994] has been contradicted by a study that did not verify a TATA function of this sequence [Chen et al., 1995]. However, the recent finding that the 1,762–1,764 mutations reduce the transcription of precore RNA by disturbing the binding of transcription factors [Buckwold et al., 1996] favors the proposition that HBeAg-related immune escape is indeed the mechanism behind the mutation. The recent presentation of a branched RNA stem loop in the 3′ core promoter/precore region and the predicted effects on the conformation of this stem-loop by the 1,762/1,764 mutations [Kidd and Kidd-Ljunggren, 1996] may contribute to explaining their emergence, because the mutations radically change the predicted base pairing of the stem loop, including the DR1 sequence. However, the existence and relevance of these effects require experimental support.

Effects of the T-1,762 mutation, which are unrelated to transcriptional regulation and RNA structure, might also be of importance. The AAG GTC→ATG ATC mutations (nt 1,762–1,764; underlined) induce amino acid (aa) substitutions at aa 130–131 in the X protein from lysine-valine to methionine-isoleucine. Thus, the exchange of aa 130–131 might be important by influencing the function of the X protein.

In the $HBeAg^+$ carriers, we found that an A→T mutation at nt 1,762 was associated with more liver damage. Because both the driving force for the mutation and the cause of liver damage is considered to be the immune response, this association is logical. Detection of the T-1,762 mutation may be useful for selecting patients suitable for therapy, because interferon treatment is likely to be successful only if the patient has entered the immunoactive stage, as supported by the findings in a recent study [Kanai et al., 1996].

Our results are in agreement with a study in which the G→A mutation at nt 1,764 was detected with a restriction enzyme technique by using the Sau3AI site created by the mutation [Takahashi et al., 1995]. In that study, the A-1,764 mutation was associated with higher alanine aminotransferase (ALT) levels and was found to be more common in carriers with chronic liver disease compared with $HBsAg^+$ blood donors. An association between the 1,762–1,764 mutations and higher ALT levels was also found in a more recent study [Kidd-Ljunggren et al., 1997]. However, due to the frequent fluctuations in ALT levels, particularly in patients with remitting disease, the absence of histological data is a limitation with these studies. Therefore, further study, including histological analyses, is required to establish the association of promoter mutations and liver damage in $HBeAg^-$ stage.

Genotyping showed that C-1,858, which prevented emergence of the precore TAG mutation, was found in genotype A, as described previously [Li et al., 1993], but was also found in East Asian carriers with genotype C. Unexpectedly, we also found that T-1,762 mutants were significantly more frequent in genotype C compared with genotype B, which is also prevalent in East Asian carriers. The reason for this difference and whether it has any impact on the course of infection are unclear. The mean age did not differ between the groups, contradicting the possibility that a difference in duration of infection would explain the finding.

The CPRS technique for mutation analysis proved to be robust and reliable. It can be applied to any point mutation, because it requires only that a restriction site is included in the primer relatively close to the 3′ end. The method did not require the sometimes diffi-

cult calibration of reaction conditions required for ordinary allele-specific PCR, and it was relatively insensitive to differences in template concentration. It may prove particularly useful in genotyping, in which it may add primer specificity to traditional RFLP typing.

Our data encourage further study on the clinical importance of the T-1,762 mutation. In particular, it is important to establish whether detection of this mutation is useful for predicting interferon response and for estimating the risk for progressive liver disease. Moreover, the biological effects of promoter mutations need to be clarified to understand the mechanism behind their emergence.

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